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ORIGINAL RESEARCH

Tadalafil attenuates spinal cord injury induced oxidative organ damage in rats

T. Emre ŞENER, İlker TINAY, Cem AKBAL, Mehmet ERŞAHİN, Özge ÇEVİK, Selin ÇADIRCI, Özlem Tuğçe ÇİLİNGİR, Şule ÇETİNEL, Göksel ŞENER

ABSTRACT

Spinal cord injury (SCI) has been shown to cause systemic inflammatory response syndrome (SIRS) which damages multiple organs due to an influx of inflammatory cells from the circulation. In this study, we evaluated the effect of tadalafil, a phoshodiesterase inhibitor, against spinal cord, kidney and bladder damage in experimental animal model of spinal cord injury. Male Wistar albino rats were divided into sham-operated control, and either vehicle or tadalafil-treated SCI groups. In order to induce SCI, a standard weight-drop method that induced a moderately severe injury (100 g/cm force) at T10, was used. Injured animals were given either 10 mg/kg tadalafil or vehicle 15 minutes post injury and repeated daily for seven days. After decapitation spinal cord, kidney and bladder tissue samples were obtained to examine oxidative tissue injury; malondialdehyde (MDA) and glutathione (GSH) levels, and superoxide dismutase (SOD), myeloperoxidase (MPO) and caspase-3 activities. Tissues were also examined histologically. In the injured animals, MDA levels MPO and caspase-3 activities in tissues were found significantly increased while tadalafil treatment reversed these increases. On the other hand SCI-induced decreases in GSH levels and SOD activities were also reversed with tadalafil treatment. According to the results, tadalafil exerts beneficial effects against SCI-induced oxidative damage in spinal cord and also in remote organs such as kidney and bladder tissues through its anti-inflammatory and antioxidant effects.

Key words: tadalafil; spinal cord injury; anti-inflammatory; spinal cord, kidney; bladder.

T. Emre Şener, İlker Tinay, Cem Akbal,
Marmara University, School of Medicine, Department of Urology, İstanbul

Mehmet Erşahin,
İstanbul Medeniyet University, School of Medicine, Department of Neurosurgery, İstanbul

Özge Çevik,
Cumhuriyet University, School of Pharmacy, Department of Biochemistry, Sivas

Selin Çadırcı, Göksel Şener
Marmara University, School of Pharmacy, Department of Pharmacology, İstanbul

Özlem Tuğçe Çilingir, Şule Çetinel
Marmara University, School of Medicine, Department of Histology & Embriology, İstanbul

Corresponding author:
Göksel Sener, Ph.D.
Marmara University, School of Pharmacy
Tıbbiye Cad. 34668 İstanbul- TURKEY
Tel: 00 90 216 414 29 62
Fax: 00 90 216 345 29 52
E-mail: gsener@marmara.edu.tr
gokselsener@hotmail.com

INTRODUCTION

Spinal cord injury (SCI) is classified as a primary injury that includes the focal mechanical damage of neural tissue and secondary degeneration process that involves a cascade of biochemical, molecular, and cellular changes. Secondary injury produces more extensive damage, and is potentially susceptible to therapeutic intervention with neuroprotective agents (1). Cellular apoptosis, increased release of excitatory amino acids, and enhanced generation of reactive oxygen species (ROS) with subsequent lipid peroxidation (LP) comprise the major complex pathway of SCI-induced secondary damage (2). Since inflammation and oxidative stress are major factors exacerbating post-SCI pathogenesis, agents that posses anti-inflammatory and antioxidant effects has been thought to provide protection against posttraumatic spinal cord injury (3-5).

Tadalafil a selective inhibitor of phosphodiesterase-5 (PDE5 I) catalyzes the breakdown of cyclic guanosine monophosphate (cGMP), one of the primary factors causing smooth muscle relaxation. Its potent action is to enhance nitric oxide-driven cGMP accumulation and to ensue vasodilatation in corpus cavernosum (6). Tadalafil and other PDE5 Is are widely used for treating erectile dysfunction in men. Recently, there are several studies on PDE-5 Is documenting their possible therapeutic applications in diseases other than erectile dysfunction (7-9**).** Furthermore, in an invitro study sildenafil was examined in adult mouse ventricular myocytes exposed to ischemia/reperfusion (I/R)

and was shown that the drug may have possible therapeutic potential in preventing myocyte cell death (10). Similarly, the protective potential of tadalafil was shown in experimental models of renal IR-injury and cyclophosphamide (CP)-induced hemorrhagic cystitis (11, 12).

Based on above findings, the experiment reported in the present study has been designed to determine whether spinal cord trauma causes multi organ damage (like in renal and bladder tissues beside spinal cord) and tadalafil could attenuate these injuries.

MATERIALS AND METHODS

Male Wistar albino rats (250-300 g) supplied by the Marmara University (MU) Animal Center (DEHAMER) were housed in an air-conditioned room with 12:12 light: dark cycles, where the temperature (22±2ºC) and relative humidity (65-70%) were kept constant. All experimental protocols were approved by the MU Animal Care and Use Committee.

Rats were divided into three groups of 8 rats in each group; 1) control group that underwent sham surgery and received 1 ml peanut oil as vehicle; 2) SCI group that underwent surgery for SCI induction and was given vehicle; 3) SCI-induced and tadalafil (10 mg/kg/day, mixed with peanut oil, orally) treated group. Treatments were started following the SCI inductions and continued for 7 days.

Induction of SCI

Anesthetized (ip ketamine and chlorpromazine; 100 mg/kg and 1 mg/kg, respectively) rats were positioned on a thermistor-controlled heating pad in a prone position and a rectal probe was inserted. Under sterile conditions, following T5-12 midline skin incision and paravertebral muscle dissection, spinous processes and laminar arcs of T7-10 were removed. The dura was left intact. Modified weight-drop model was performed for SCI (13). The animals were subjected to an impact of 100 g/cm (10 g weight from 10 cm height) to the dorsal surface of the spinal cord. The force was applied via a stainless stell rod (3 mm diameter tip) that was rounded at the surface. The rod was dropped vertically through a 10 cm guide tube that was positioned perpendicular to the center of the spinal cord. Afterward, the muscles and the incision were sutured. Following surgical procedure, the rats were placed in warming chamber and their body temperatures were maintained at approximately 37 °C until they were completely awake.

A week after SCI induction, rats were decapitated to obtain spinal cord, kidney and bladder tissue samples for the biochemical and histological analysis.

Measurement of tissue Myeloperoxidase Activity

Myeloperoxidase (MPO) activity in tissues was measured by a procedure similar to that described by Hillegas et al. (14). Spinal cord tissue samples were homogenized in 50 mM potassium phosphate buffer with a pH of 6.0, and centrifuged at 41,400 g for 10 min. The pellets were then suspended in 50 mM phosphate buffer containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw-cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance, measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of tissue malondialdehyde (MDA) and glutathione (GSH) levels

Bladder tissue samples were homogenized with ice-cold 150 mM KCl for the determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (15). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (16). Briefly, after centrifugation at 3000 rev./min for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. Results are expressed in µmol GSH/g tissue.

Measurement of tissue superoxide dismutase (SOD) activities

SOD activity in the bladder tissue samples was measured according to the previously described method (17). Briefly, measurements were performed in cuvettes containing 2.8 mL 50 mM potassium phosphate (pH=7.8) with 0.1 mM EDTA, 0.1 mM 0.39 mM riboflavin in 10 mM potasium phosphate (pH 7.5), 0.1 mL of 6 m M O-dianisidin.2 HCl in deionized water, and tissue extract (50, 100 mL). Cuvettes with all their components were illuminated with 20-W Slylvania Grow Lux fluorecent tubes that were placed 5 cm above and to one side of cuvettes maintaining a temperature of 37º C. Absorbances were measured at 460 nm with Schimadzu UV-02 model spectrophotometer. A standard curve was prepared routinely with bovine SOD (Sigma Chemical Co, S-2515-3000 U) as reference. Absorbance readings were taken at 0 and 8 min of illumination and the net absorbances were calculated.

Measurement of tissue caspase-3 activity

Caspase-3 cellular activity assay kit (Calbiochem, San Diego, CA) and assay kit (EnzyChrom, BioAssay Systems, Hayward CA, USA) was used. Enzymes activities were presented as nmol /min/ mg protein.

Histopathological analysis

For light microscopic investigations, tissues were fixed in %10 formaldehyde solution and underwent routine histologic preparation and were embedded in paraffin. Paraffin tissue blocks were sectioned 5µm thickness on a rotary microtome and mounted on glass-slides. Sections of kidney and urinary bladder were stained with hematoxylin and eosin (H&E), sections of spinal cord were stained with luxol fast blue and cresyl violet (LFB&CV). Histologic sections were examined under Olympus BX51 Photomicroscope for characterization of histopathological changes.

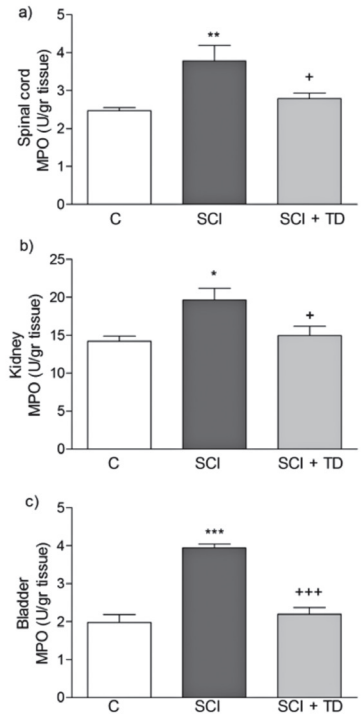

Figure 1. Myeloperoxidase (MPO) activity in the a) spinal cord, b) kidney and c) bladder tissues of sham-operated control (C) and vehicle- or tadalafil-treated spinal cord injury (SCI) groups. Each group consists of 10 rats. Values are represented as mean ± SEM. * p<0.05, **p<0.01, *** p<0.001; vs control group; $^{+}$p<0.05, $^{+++}$p<0.001; vs vehicle-treated SCI group.

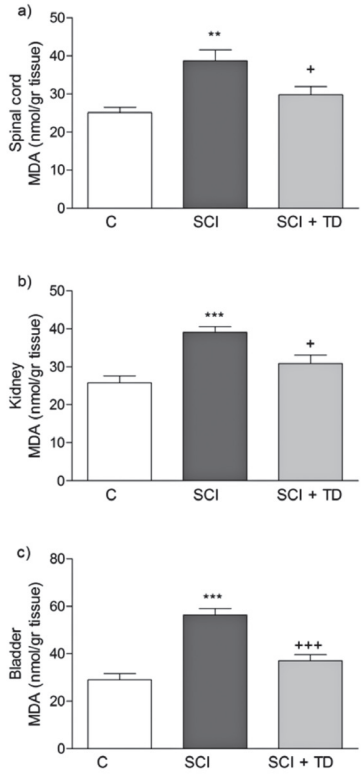

Figure 2. Malondialdehyde (MDA) levels in the a) spinal cord, b) kidney and c) bladder tissues of sham-operated control (C) and vehicle- or tadalafil-treated spinal cord injury (SCI) groups. Each group consists of 10 rats. Values are represented as mean ± SEM. **p<0.01, *** p<0.001; vs control group; $^{+}$p<0.05, $^{+++}$p<0.001; vs vehicle-treated SCI group.

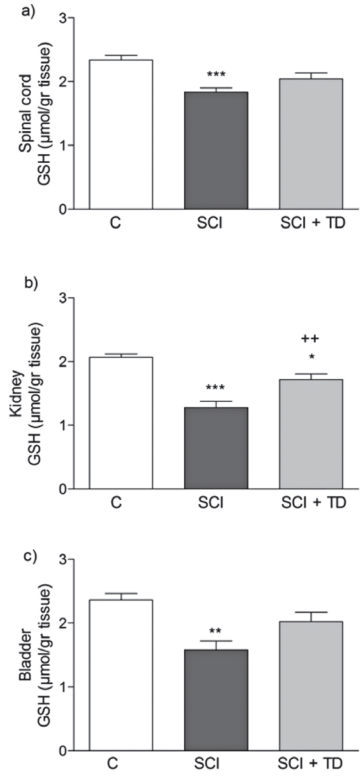

Figure 3. Glutathione (GSH) levels in the a) spinal cord, b) kidney and c) bladder tissues of sham-operated control (C) and vehicle- or tadalafil-treated spinal cord injury (SCI) groups. Each group consists of 10 rats. Values are represented as mean ± SEM. * p<0.05, **p<0.01, *** p<0.001; vs control group; $^{++}$p<0.01; vs vehicle-treated SCI group.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4·0 (GraphPad Software, San Diego, CA, USA). All data are expressed as means ± SEM. Groups of data were compared by ANOVA followed by Tukey's multiple comparison tests. Values of p<0.05 were regarded as significant.

RESULTS

As a result of SCI-induced oxidative stress, MPO activity (Fig.1) and MDA levels (Fig.2) in spinal cord, kidney, and bladder tissues were found to be significantly (p<0.05-0.001) higher than that of the control group. In the SCI-damaged rats that received tadalafil treatment, MPO activities and MDA levels were significantly reduced (p<0.05-0.001), with the levels being near the values found in the control group.

In accordance with the increased oxidative stress, GSH levels were found to be significantly depleted in the all the mentioned tissues. On the other hand tadalafil treatment preserved the GSH levels only in kidney tissues significantly (Fig.3). Similarly SOD activities which were also depleted due to SCI (p<0.01-0.001), were significantly preserved with tadalafil treatment (p<0.05-0.001, Fig.4).

Caspase-3 activity of spinal cord, kidney and bladder tissues as an index of apoptosis was significantly elevated in the vehicle-treated SCI damaged rats (p<0.001; Fig. 5). This SCI mediated rise in apoptosis was significantly depressed in the spinal cord and bladder tissues after tadalafil treatment (p<0.01-0.001).

Histological results

Spinal cord: Control group showed no evidence of myelin damage (Fig. 6a). However in the spinal cords of SCI induction group, decreased staining intensity was clear. When compared with control group SCI resulted in severe degeneration of white matter. White matter was observed with intensive vacuole formation (Fig. 6b). In the tadalafil treated SCI group, the spinal cords of the animals exposed nearly normal staining intensity and less vacuol formation was observed (Fig. 6c).

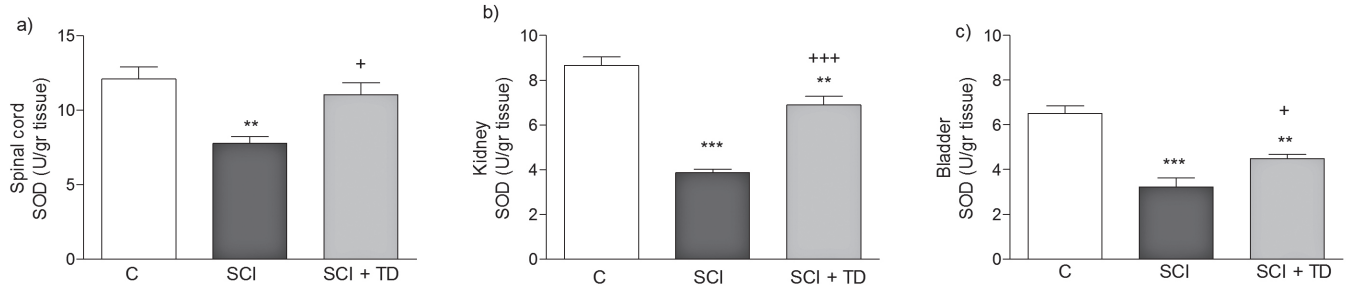

Figure 4. Superoxide dismutase (SOD) activity in the a) spinal cord, b) kidney and c) bladder tissues of sham-operated control (C) and vehicle- or tadalafil-treated spinal cord injury (SCI) groups. Each group consists of 10 rats. Values are represented as mean ± SEM. **p<0.01, *** p<0.001; vs control group; $^{+}$p<0.05, $^{+++}$p<0.001; vs vehicle-treated SCI group.

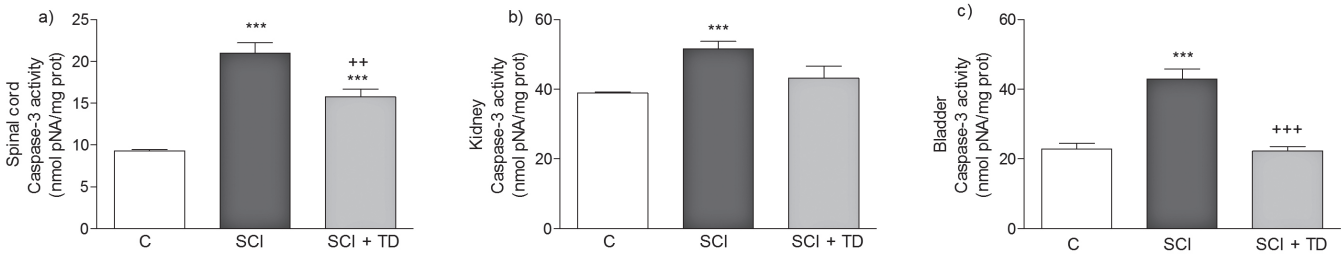

Figure 5. Caspase-3 activity in the a) spinal cord, b) kidney and c) bladder tissues of sham-operated control (C) and vehicle- or tadalafil-treated spinal cord injury (SCI) groups. Each group consists of 10 rats. Values are represented as mean ± SEM. *** p<0.001; vs control group; $^{++}$p<0.01, $^{+++}$p<0.001; vs vehicle-treated SCI group.

Kidney: Control renal tissue demonstrated a regular alignment of both glomerular and tubular structures (Fig. 7a), whereas in the SCI group a severe interstitial edema, tubular dilation and glomerular congestion were the prominent features (Fig. 7b). Tadalafil treatment of SCI induction had led to a marked regeneration of renal morphology with glomerular, tubular and interstitial structures (Fig. 7c).

Urinary bladder: Control urinary bladder was observed with regular layout of epithelium, lamina propria and muscle layer (Fig. 8a). SCI induction resulted with marked distrophy of muscle tissue and prominent edema in lamina propria (Fig. 8b). Tadalafil induction showed a regenerative effect in muscle tissue and reduced edema in lamina propria (Fig. 8c).

DISCUSSION

In this study, we observed that SCI resulted in a significant oxidative damage in spinal cord, kidney and bladder tissues, as evidenced by increased lipid peroxidation with a concomitant decrease in glutathione level and SOD activity. The oxidative damage and tissue neutrophil accumulation due to SCI were attenuated by tadalafil treatment. Furthermore, histological data are also in accordance with the biochemical changes.

It has been previously demonstrated that SCI causes local and systemic inflammatory responses which are associated with the production of free radicals (18-20). Free radical-induced lipid peroxidation is one of the mechanisms of the secondary spinal cord injury which result in an autodestructive phenomenon of spinal cord (21). In the current study, we monitored the ROS-induced tissue damage via MDA levels, an end product of lipid peroxidation, and found that SCI caused a significant increase in MDA levels not only in the spinal cord tissue but also in kidney and bladder tissues. Furthermore our results also demonstrated that inflammatory response is also an important factor in the development of secondary injury following SCI. Activated neutrophils exacerbate tissue injury through the production of oxygen metabolites and the activation of cytotoxic enzymes including MPO (22). Herein, the presence of increased neutrophil accumulation, as assessed by elevated MPO activity in the spinal cord, kidney and bladder tissues indicated that SCI-induced oxidative injury in these tissues involves a contribution by neutrophil accumulation. In our previous study we have demonstrated increased MPO activity not only in spinal cord tissues but also in bladder and corpus cavernosum tissues in spinal cord injured rats (23, 24). Wang et al, have demonstrated that SCI-induced oxidative stress increased MPO activity in spinal cord tissues in their spinal cord ischemia/reperfusion model (25). In the present study PDE5 inhibitor, tadalafil effectively reduced this enzyme activity suggest that tadalafil could exert anti-inflammatory effects. The ability of tadalafil to inhibit neutrophil accumulation and the associated MPO activity is demonstrated in literature. In the study of Kucuk et al increased MPO activity and

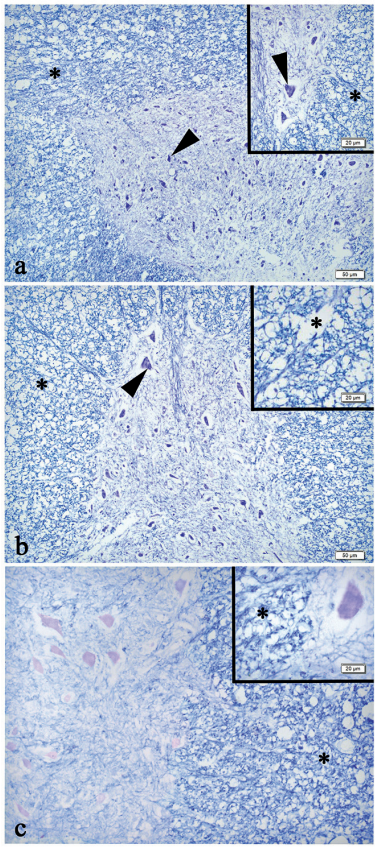

Figure 6. LFB-CV stained sections of spinal cords. a. Control group: Normal staining intensity and regular morphology of neurons in gray matter (arrowheads) and white matter (asterisk), b. Vehicle-treated SCI group: Prominent vacuol formation in white matter (asterisk), and neurons in gray matter (arrowhead) c. Tadalafil- treated SCI group: Reduced vacuol formation in white matter (asterisk). Gray matter displayed no change in the SCI and Tadalafil treated SCI groups.

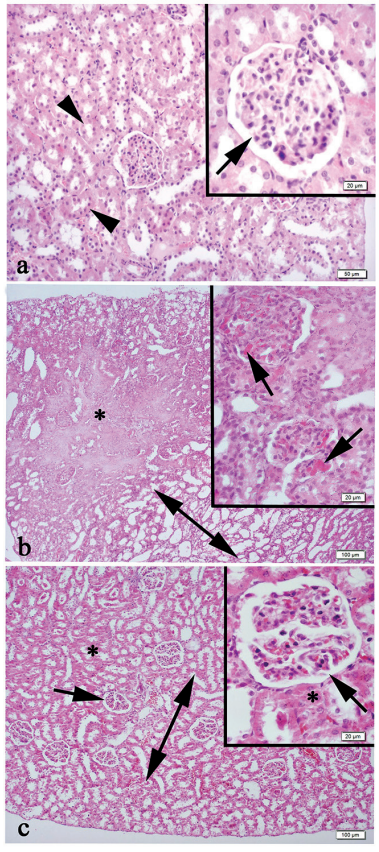

Figure 7. H&E stained sections of kidney. a. Control group: Regular contour of tubuli (arrowheads) and glomerulus (arrow). b. Vehicle-treated SCI group: Severe edema in interstitial area (asterisk) and marked tubular dilation (double-headed arrow). Note the congestion of glomeruli (arrows). c. Tadalafil-treated SCI group: Prominent regeneration in interstitial area (asterisk), glomeruli (arrows) and tubuli (double-headed arrow).

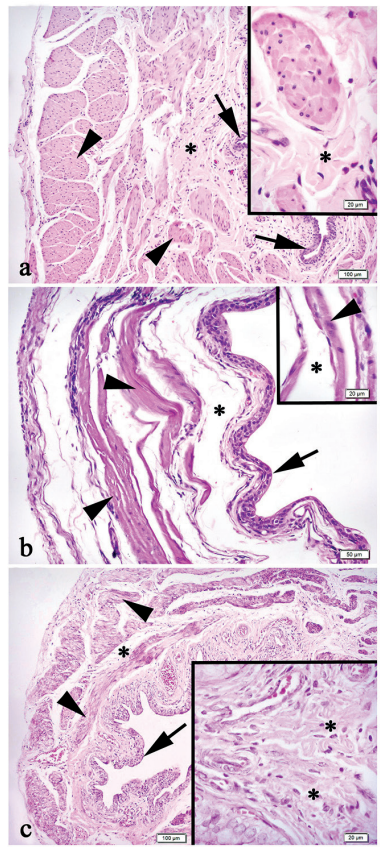

Figure 8. Micrographs of H-E stained urinary bladder sections. a. Control group: Regular epithelium (arrow), normally organized muscle layer (arrowhead) and lamina propria (asterisk). b. Vehicle-treated SCI group: Epithelium with normal morphology (arrow), highly distrophic muscle layer (arrowheads) and degenerated lamina propria (asterisk). c. Tadalafil-treated SCI group: Epithelium with regular morphology (arrow), compared with SCI group showed regeneration in muscle layer (arrowheads), lamina propria with normal architecture (asterisks).

MDA levels in renal tissues following I/R injury reversed with tadalafil treatment (26). Furthermore tadalafil has exerted beneficial effects on epigastric island flaps against I/R injury (27).

The biologically active form, reduced GSH, is a key contributor to the cellular antioxidant defense system and to the maintenance of the intracellular redox status for the preservation of thiol–disulfide redox states of proteins (28). It is well known that GSH provides major protection against oxidative injury, by participating in the cellular system of defence against oxidative damage and it has been reported that the tissue injury induced by various stimuli is coupled to glutathione depletion (29, 30). In the current study the decrease in glutathione levels in spinal cord injured rat tissues was probably because of its consumption during oxidative stress. Indeed antioxidative enzyme SOD was also found to be depleted. On the other hand, tadalafil treatment to the injured rats restored the SOD activity in spinal cord, kidney and bladder tissues. GSH levels were also increased after tadalafil treatment in kidney tissues significantly. In the spinal cord and bladder tissues although GSH levels were not increased significantly, these levels tended to increase since GSH levels were not significantly different than that of controls.

It has been demonstrated that GSH is also involved in cellular signaling, regulation and redox activation of transcription factors, and thiol–disulfide exchange reactions. Thus alterations in the redox status and elevated oxidative stress activating caspases might play an important role in apoptosis (31). Accordingly, apoptosis is a major complicating process related to oxidative events in the pathogenesis of secondary injury after SCI (32). In a

previous study we demonstrated that SCI causes an increase in caspase-3 activity in spinal cord and bladder tissues where MDA levels and MPO activities were also increased while GSH levels were decreased (5, 23). In agreement with this report, the current data documented that, caspase-3 activities were increased due to oxidative stress in spinal cord, kidney and bladder tissues and tadalafil decreasing neutrophil infiltration to the tissues and attenuated oxidative stress caused a depressive effect on caspase-3.

In conclusion, the findings of the present study demonstrated that increased oxidative stress due to SCI caused multiorgan damage as seen in kidney and bladder tissues in addition to the spinal cord tissue. Furthermore, our results also suggest that tadalafil treatment exert significant protective effects against SCI-induced tissue damage through their ability to inhibit neutrophil infiltration, lipid peroxidation and apoptosis and to balance the oxidant-antioxidant status.

Tadalafil sıçanlarda oluşturulan omurilik yaralanmasına bağlı oksidan doku hasarını azaltır

ÖZET: Omurilik yaralanması dolaşımdan çıkan inflamatuar hücreler aracılığı ile sistemik inflamatuar yanıt sendromuna yol açarak çoklu organ hasarına neden olur. Bu çalışmada bir fosfodiesteraz inhibitörü olan tadalafilin deneysel omurilik hasarında omurilik, böbrek ve mesane dokuları üzerine olan etkileri incelendi. Erkek Wistar albino sıçanlar taklit cerrahi ve taşıyıcı ve tadalafil tedavili omurilik yaralanması olmak üzere 3 gruba ayrıldı. Omurilik yaralanması için T10 seviyesinde standart ağırlık düşürme (100 g/cm güç) metodu kullanıldı. Omurilik yaralanması oluşturulan hayvanlara taşıyıcı ya da tadalafil (10 mg/kg) hasardan 15 dakika sonra ve takiben yedi gün boyunca her gün uygulandı. Dekapitasyondan sonra oksidan doku hasarını belirlemek amacı ile omurilik, böbrek ve mesane dokuları alınarak, malondialdehit (MDA) ve glutatyon (GSH) düzeyleri, süperoksid dismutaz (SOD), miyeloperoksidaz (MPO) ve kaspaz-3 aktiviteleri ölçüldü. Dokular ayrıca histolojik olarak da incelendi. Hasar oluşturulan hayvanlarda dokularda MDA düzeyleri ve MPO ve kaspaz-3 aktivitesi önemli şekilde arterken tadalafil tedavisi bu artışı geri çevirdi. Diğer taraftan omurilik hasarının neden olduğu GSH düzeylerinde ve SOD aktivitesinde gözlenen azalmalar tadalafil tedavisi ile geri çevrildi. Bu sonuçlara göre tadalafil antiinflamatuar ve antioksidan etkileriyle omurilik yaralanmasının neden olduğu omurilik, böbrek ve mesane dokularında oluşan oksidan hasara karşı faydalı etki göstermiştir.

Anahtar kelimeler: tadalafil, omurilik hasarı, anti-inflamatuar, omurilik, böbrek, mesane

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